Remarks

Claims 14-48 were pending. However, the Office action states that only claims 14-28 were pending. Claims 29-48 were added to the application on November 3, 2004 by a Second Preliminary Amendment. Therefore, acknowledgement of this amendment is requested.

By this amendment, claims 14, 32-34, and 36-37 are cancelled without prejudice to prosecution in a future application. Claim 30 was cancelled as redundant in view of claim 27. Claims 49-52 are added. Therefore, claims 15-29, 31, 35, and 38-52 are now pending.

Support for the new claims and claim amendments can be found throughout the specification, for example:

Claims 15, 17, 20, 21, 25, and 39-41: page 10, lines 13-14 and lines 24-25; page 11, line 13 – page 12, line 15.

Claim 22: page 10, lines 24-25 and page 13, lines 22-24.

Claims 26 and 27: re-written as independent claims.

Claims 49-51: page 13, lines 22-25.

Claim 52: page 10, lines 24-25.

The specification was amended to correct obvious typographical errors. In view of the restriction requirement in the parent application the specification was amended to clarify the title and abstract, as requested by the examiner.

No new matter is introduced by these amendments, and no amendments were made to distinguish prior art.

Applicants thank the Examiner for indicating the allowability of claims 24, 26 and 27 if written in independent form. Claim 24 is already an independent claim, and thus is allowable. Claims 26 and 27 have been amended to be independent claims, and Applicants request that they be allowed.

Telephone Interview with Examiner

Applicants thank Examiner Kruse for the courtesy of an interview with Applicants' representative Sheree Lynn Rybak, Ph.D. on January 4, 2006. All of the claim rejections were discussed. Applicants agreed to amend the title and the abstract, to reflect that this application is related to transgenic plants that express temporin peptides. Applicants also agreed to cancel one of claims 14 or 15 in view of the double patenting rejection.

With regard to the 35 U.S.C. § 112, first paragraph rejections, Examiner Kruse explained that the primary issue was that there is teaching that temporins C, D, E, H and K are inactive or have a negligible effect on pathogenic bacteria, and thus are inoperable species. However, it was agreed that temporins A, B, F, G, L are enabled species, as there is evidence that these temporins have the desired biological activity, or at least there is no evidence indicating they do not have such activity. Applicants agreed to amend the claims to remove references to temporins C, D, E, H and K, unless evidence of their having antimicrobial biological activity could be found and presented to the Examiner.

With regard to temporin variants, it was agreed that the specification enabled temporin variants, as well as methods for determining whether such variants retained antimicrobial biological activity. Applicants' representative noted that those skilled in the art have made and tested temporin variants, such as variants of temporin A (for example see Rollins-Smith *et al.*, *Antimicrob. Agents Chemther.* 47:1157, 2003; Exhibit A). This demonstrates that making and testing temporin variants does not require undue experimentation. Examiner Kruse agreed, and indicated that the percent identity in claim 22 could be decreased to 70% in view of the temporin variants that retain antimicrobial activity.

Objections to the Specification

The title and abstract have been amended to be descriptive of the current invention, due to the restriction in the parent application. In view of these amendments, Applicants request that the objections to the specification be withdrawn.

35 U.S.C. § 112, first paragraph

Claims 14, 15, 20, 21, 22, 23, 25 and 28 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Claims 14-23, 25 and

28 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Applicants disagree and request reconsideration.

It is Applicants understanding that the primary reason for the rejections under 35 U.S.C. § 112, first paragraph was due to the fact that some temporins (namely temporins C, D, E, H, and K) have been shown to be inactive or have negligible effects on pathogenic bacteria. Applicants have amended the claims to clarify that plants expressing a temporin A, B, F, G, or L peptide having the desired biological activity (such as the ability to provide the plant with resistance to bacteria or fungi or both) are claimed.

The specification provides support for transgenic plants that express native temporin peptides, as well as variants thereof. Native temporin A, B, F, G and L peptide sequences are provided in SEQ ID NOS: 18, 19, 23, 17, and 26, respectively. It is noted on page 16, lines 20-25 of the specification that nucleic acid sequences encoding such peptides can be determined using the genetic code or from a publicly available cDNA or genomic sequence known in the art.

In addition, the specification provides substitutions that can be made to these sequences, as well as methods to determine which variants retain the desired biological activity (such as the ability to provide bacterial or fungal disease resistance to the plant in which the peptide is expressed). For example, beginning on page 17, line 8, the specification describes how other sequences can be added to a temporin peptide (for example via the N-terminus). In addition, beginning on page 19, line 5 of the specification, a description of variants is provided. Table 1 on page 20 provides specific examples of conservative amino acid substitutions that can be made to a native temporin sequence.

Methods of introducing the variant temporin sequence into a plant, and determining its ability to provide disease resistance, are provided on page 11, line 13- page 12, line 15; page 21, line 15 – page 26, line 2; page 30, line 30 - page 31, line 10; and page 32, line 21- page 34, line 21.

It does not require undue experimentation to make temporin variants and determine their antimicrobial activity. This is demonstrated by Rollins-Smith *et al.*, who made and tested multiple temporin A variants, and determined the ability of such variants to inhibit fungal growth. Table 1 of Rollins-Smith *et al.* shows the variants tested. For example, temporin A from *Rana temporaria* and from *Rana pipiens* differ by 5 amino acids (about 61% sequence identity). The specific amino acid changes are conservative changes. That these specific

conservative substitutions could be made to a temporin sequence is provided in Table 1 on page 20 of the specification. Other variants having even less sequence identity (such as I4G10 and I4S10, which have about 46% and 54% sequence identity, respectively, to temporin A from *Rana temporaria*) had very similar antimicrobial activity to the native sequence (see page 1159, second column, first full paragraph). In view of the observation that variants of temporin with at least 70% sequence identity have been shown to retain antimicrobial activity, the claims are enabled by the application.

In view of the amendments to clarify that plants expressing temporin molecules having temporin biological activity are claimed, and the arguments presented above, Applicants request that the 35 U.S.C. § 112, first paragraph, written description and enablement rejections be withdrawn.

If any issues remain before a Notice of Allowance is issued, the Examiner is invited to telephone the undersigned.

Respectfully submitted,

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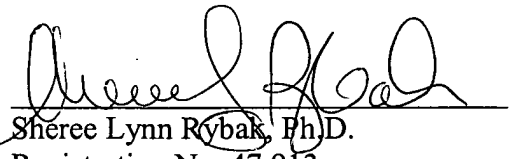
By 
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Activities of Temporin Family Peptides against the Chytrid Fungus (*Batrachochytrium dendrobatidis*) Associated with Global Amphibian Declines

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Temporin A and structurally related peptides produced in amphibian dermal granular glands and in wasp venom were tested for growth inhibition of *Batrachochytrium dendrobatidis*, a pathogen associated with global amphibian declines. Two natural amphibian temporins, a wasp temporin, and six synthetic analogs effectively inhibited growth. Differences in potency due to amino acid substitution suggest that ability to penetrate membranes and form an α-helical structure is important for their effectiveness against this pathogen.

Global amphibian declines (reviewed in references 4, 6, and 10) have been associated with a novel chytrid fungus (*Batrachochytrium dendrobatidis*) that infects the skin (2, 3, 15, 19). Among the innate defenses employed by amphibians to resist skin infections is the production of antimicrobial peptides in dermal granular glands (reviewed in references 17, 24, and 33). Our previous studies have shown that 17 antimicrobial peptides representing 11 families of peptides inhibit growth of *B. dendrobatidis*. Six are active against a second fungal pathogen (*Basidiobolus ranarum*) associated with declines of the Wyoming toad, and two can inhibit in vitro plaque formation by iridoviruses pathogenic to amphibians and fish (5, 20–22). One of the peptides with antichytrid activity was a member of the temporin family (temporin 1Ob from *Rana ornativentris*) (20). For the present study, we have determined the antichytrid activity of several other members of this peptide family. Temporins, originally isolated from the European red frog, *Rana temporaria* (25), and later isolated from a number of North American and European ranid species (7–9, 11, 12, 23), are linear peptides containing 10 to 14 amino acids. All are α-amidated at their carboxyl-terminal ends. They occur not only in the skin secretions of a number of amphibians but also in wasp venom (reviewed in reference 27). They are most active against gram-positive bacteria, but some show activity against gram-negative bacteria and two have been shown to be active against the fungus *Candida albicans* (7–9, 11, 12, 25, 28). We show here that three additional natural temporins (two from amphibians and one from a wasp) and six synthetic analogs of temporin A (TA) can inhibit growth of *B. dendrobatidis*. Differences in potency due to amino acid substitution suggest that the likely mechanism of action against this pathogen is attachment to the membrane, followed by the folding of the temporins into an α-helical structure that facilitates disruption of the membrane. The effectiveness of the peptides may also depend on their ability to resist fungal proteases.

B. dendrobatidis was cultured and peptide inhibition of

TABLE 1. Natural and synthetic temporin-like peptides[a]

Peptide	Amino acid sequence[f]	No. of amino acids	NC[g]	%H[h]
TA[b]	F-L-P-L-I-G-R-V-L-S-G-I-L-NH_2	13	+2	61
T-1P[c]	F-L-P-I-V-G-K-L-L-S-G-L-L-NH_2	13	+2	61
Rana-6[d]	F-I-S-A-I-A-S-M-L-G-K-F-L-NH_2	13	+2	69
VesCP-M[e]	F-L-P-I-I-G-K-L-L-S-G-L-L-NH_2	13	+2	61
DTA	f-l-p-l-i-g-r-v-l-s-g-i-l-NH_2	13	+2	61
LDTA	F-l-P-l-I-G-R-v-L-s-G-i-L-NH_2	13	+2	61
W1-TA	W-L-P-L-I-G-R-V-L-S-G-I-L-NH_2	13	+2	54
I4G10	F-L-P-I-I-A-S-L-L-G-K-L-L-NH_2	13	+2	69
I4S10	F-L-P-I-I-A-S-L-L-S-K-L-L-NH_2	13	+2	69
CATA	K-W-K-L-F-K-K-L-P-L-I-G-R-V-L-NH_2	15	+6	47

[a] Adapted from Table 2 of reference 28.
[b] *R. temporaria*.
[c] *Rana pipiens*.
[d] *Rana catesbeiana*.
[e] *Vespa mandarinia*.
[f] NH_2, amide (-CO-NH_2). D amino acids are in lowercase; Gly is nonchiral.
[g] NC, net charge at pH 7.
[h] %H, percentage of hydrophobic residues (average, 61%) based on the scale of Kyte and Doolittle (14).

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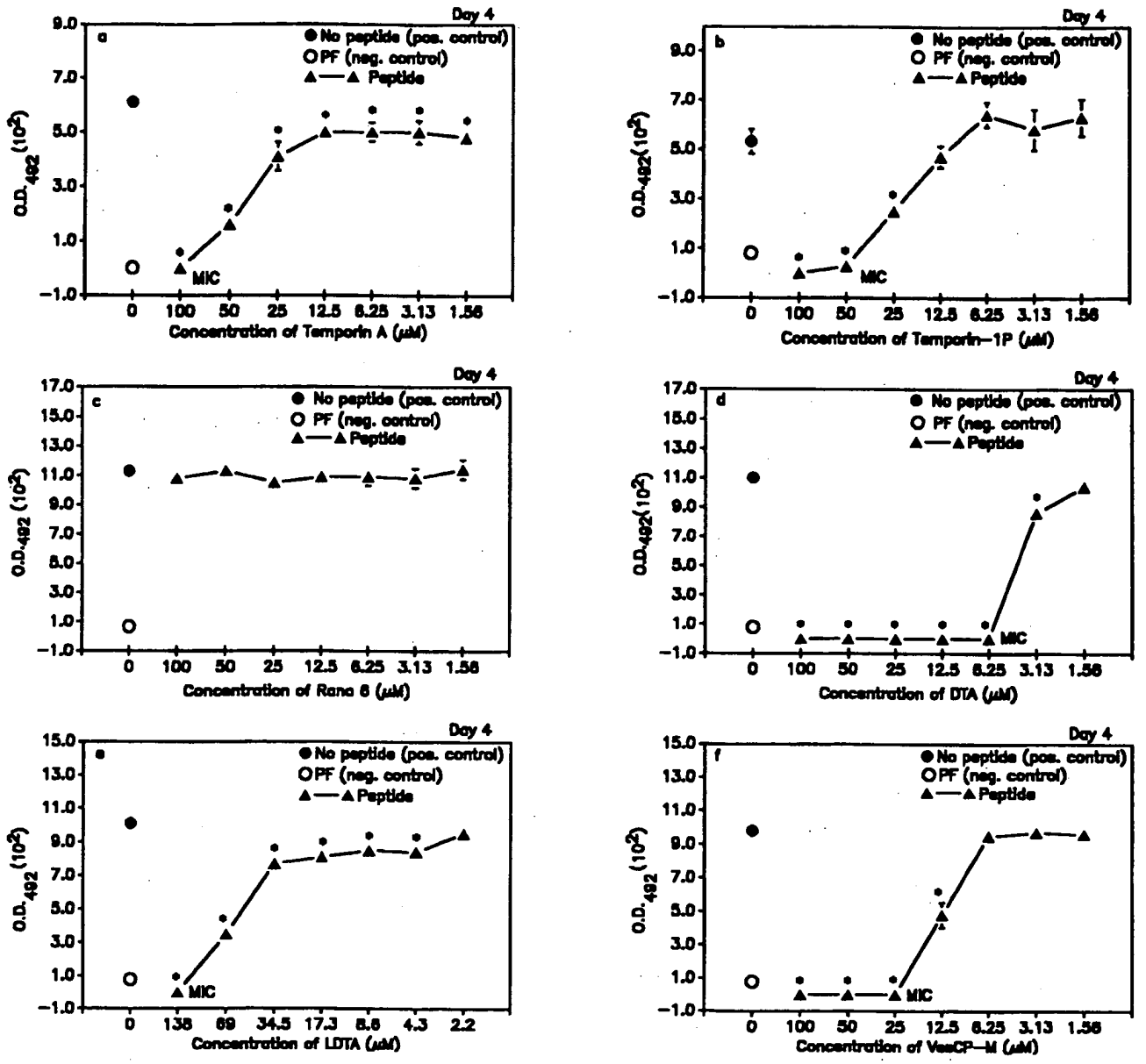

FIG. 1. Growth inhibition of *B. dendrobatidis* at 4 days of culture by TA (a), T-1P (b), Rana-6 (c), DTA (d), LDTA (e), and VesCP-M (f). Each data point represents the mean ± standard error (SE) of three or more replicate wells. Where no error bar is shown, the SE was less than the diameter of the symbol. *, significantly less growth than positive controls (one-tailed Student *t* test; $P \leq 0.05$). PF, paraformaldehyde. The results are representative of two assays for each peptide except LDTA and VesCP-M, which were assayed once. Each peptide was also assayed at least once for inhibition of zoospore-enriched cultures (see Table 2). MIC is the lowest concentration at which no growth was detected. $O.D._{492}$, optical density at 492 nm.

chytrid growth was assayed as previously described (20–22). MIC is defined as the lowest concentration at which no growth was detectable. The peptides examined in these experiments are listed in Table 1. Shown are the amino acid sequences, species of origin, numbers of amino acids, net charges at pH 7, and percentages of hydrophobic residues. All peptides were synthesized by solid-phase techniques using 9-fluorenylmethoxy carbonyl chemistry as previously described (28). The peptides were purified by reverse-phase high-pressure liquid chromatography and characterized by amino acid analysis and electrospray ionization mass spectrometry. All peptides were dissolved in glass-distilled water, filter sterilized, frozen in small aliquots at high concentration and used at various dilutions for culture.

The activities of three natural amphibian temporins as inhibitors of growth of mature cells of *B. dendrobatidis* are shown in Fig. 1a to c, and MICs are shown in Table 2. TA and temporin 1-P (T-1P) significantly inhibited growth at concentrations above 25 μM (35 μg/ml). Ranatuerin-6 (Rana-6) showed little activity against mature chytrid cells but did weakly inhibit growth of zoospores at a concentration above 50 μM (70 μg/ml) (data not shown). It is not yet clear why Rana-6 had

TABLE 2. MICs necessary to completely inhibit growth of *B. dendrobatidis* (mature cells or zoospores)

Peptide	Species of origin	MIC against mature cells at day 4		MIC against zoospores at day 5	
		μM	μg/ml	μM	μg/ml
TA	*Rana temporaria*	100	140	66	92
T-1P	*Rana pipiens*	50	68	50	68
Rana-6	*Rana catesbeiana*	>100	>140	>100	>140
DTA	Synthetic	6.25	8.7	6.25	8.7
LDTA	Synthetic	138	193	>25	>35
VesCP-M	*Vespa mandarinia*	25	34.6	6.25	8.6
W1-TA	Synthetic	36	52	25	36
I4G10	Synthetic	57	80	29	40
I4S10	Synthetic	100	143	32	46
CATA	Synthetic	100	184	100	184

significantly reduced activity in comparison with other members of the temporin family. All of the amino acid differences are conservative changes with respect to the hydrophobicity or hydrophilicity of the position.

TA is composed entirely of L amino acids. In comparison, an all-D isomer, designated DTA, had significantly greater potency in the inhibition of growth of *B. dendrobatidis* (Fig. 1d and Table 2). Antimicrobial peptides are thought to act independently of specific membrane receptors, and all-D isomers are predicted to have activities very similar to those of the naturally occurring all-L-isomer forms (29). We speculate that the enhanced activity of DTA may be due to its stability against proteolytic enzymes produced by *B. dendrobatidis*. Although specific proteolytic enzymes have not been identified or characterized for this species, preliminary studies suggest that secreted products of growing *B. dendrobatidis* can degrade casein and gelatin (J. Piotrowski, S. Annis, and J. E. Longcore, unpublished observations).

The TA isomer designated LDTA has the same amino acid sequence as TA and DTA, but alternate amino acids (amino acids 2, 4, 8, 10, and 12) are of the D configuration. Natural TA has been shown to adopt an α-helical conformation in aqueous solutions of trifluoroethanol, a model system for the hydrophobic membrane environment (31). LDTA is predicted to be incapable of forming an α-helix (28), although it might form a larger-diameter helix such as that found in gramicidin A (13). An α-helical conformation has been shown to be required for the antimicrobial activities of many antimicrobial peptides. Therefore, the activity of LDTA is predicted to be less than that of TA, and this prediction was confirmed experimentally (Fig. 1e and Table 2). This is persuasive evidence that the ability of temporins to assume an α-helical conformation is important for their activity against *B. dendrobatidis*.

Another natural analog of TA, which is found in the venom of the wasp *Vespa mandarinia* (VesCP-M) (27, 28), and a second, synthetic analog of TA in which the amino-terminal phenylalanine is replaced by tryptophan (W1-TA) had significantly better activity for inhibiting the growth of *B. dendrobatidis* than TA (Fig. 1f and Table 2). Similar results were obtained with bacteria as targets (30). Substitution of the amino-terminal tryptophan for phenylalanine in the analog W1-TA may enable the peptide to insert itself into membranes more freely.

The peptide designated CATA is a hybrid composed of the amino-terminal sequence (residues 1 to 7) of cecropin A, an antimicrobial peptide originally isolated from the hemolymph of pupae of the silk moth *Hyalophora cecropia* (26), and residues 2 to 9 of TA. Its antimicrobial activity profile and MIC against *B. dendrobatidis* are similar to those of TA and other natural amphibian temporins (Table 2).

The analogs I4G10 and I4S10 were synthesized to match two possible consensus amino acid sequences derived by comparison of the amino acid sequences of 30 temporin-like peptides found in the skin secretions of various amphibian species (32). Their antimicrobial activity profiles and MICs against *B. dendrobatidis* are very similar to those of the natural temporins (Table 2).

Studies of replication and transmission of *B. dendrobatidis* suggest that spread of infection from one area of the skin to another or to a new host occurs by means of motile zoospores. Thus, the effectiveness of each antimicrobial peptide against the zoospore stage was tested to determine whether the peptide could inhibit infections. A comparison of the MICs for purified zoospores versus those for mature cells shows that, for most peptides tested, zoospores were completely inhibited at a concentration that was an average of 66% of that necessary to completely inhibit mature cells (Table 2).

The mechanism(s) of action of these peptides is unknown. Possible mechanisms include formation of pores in microbial bilayer membranes and membrane solubilization by a "carpet-like" mechanism that leads to a disruption in the internal homeostasis of the cell and death (1, 18). The first step in such a process would be the binding of the peptide to the cell membrane. For *B. dendrobatidis*, the predominant mode of binding does not appear to be via electrostatic interactions between the positively charged peptides and negatively charged membrane phospholipids because CATA, the peptide with the greatest net positive charge (+6), was not as active as many of the other peptides of Table 1 that are less positively charged (+2). It may be that the major mode of binding to the membrane of this organism is through hydrophobic interactions, as has been suggested for bacteria (16).

Because amphibian species are threatened on a global scale, further research is urgently needed to understand the role of temporins and other antimicrobial peptides in the innate defense capacity of amphibians.

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